NPY/NPF-Related Neuropeptide FLP-34 Signals from Serotonergic Neurons to Modulate Aversive Olfactory Learning in Caenorhabditis elegans

Melissa Fadda
Nathan De Fruyt
Charline Borghgraef
Jan Watteyne
Katleen Peymen

See next page for additional authors

Follow this and additional works at: https://ro.uow.edu.au/ihmri

Part of the Medicine and Health Sciences Commons

Recommended Citation
Fadda, Melissa; De Fruyt, Nathan; Borghgraef, Charline; Watteyne, Jan; Peymen, Katleen; Vandewyer, Elke; Naranjo Galindo, Francisco; Kieswetter, Amanda; Mirabeau, Olivier; Chew, Yee Lian; Beets, Isabel; and Schoofs, Liliane, "NPY/NPF-Related Neuropeptide FLP-34 Signals from Serotonergic Neurons to Modulate Aversive Olfactory Learning in Caenorhabditis elegans" (2020). Illawarra Health and Medical Research Institute. 1543.
https://ro.uow.edu.au/ihmri/1543

Research Online is the open access institutional repository for the University of Wollongong. For further information contact the UOW Library: research-pubs@uow.edu.au
NPY/NPF-Related Neuropeptide FLP-34 Signals from Serotonergic Neurons to Modulate Aversive Olfactory Learning in Caenorhabditis elegans

Abstract
Copyright © 2020 the authors. Aversive learning is fundamental for animals to increase chances of survival. In addition to classical neurotransmitters, neuropeptides have emerged to modulate such complex behaviors. Among them, neuropeptide Y (NPY) is well known to promote aversive memory acquisition in mammals. Here we identify an NPY/neuropeptide F (NPF)-related neuropeptide system in Caenorhabditis elegans and show that this FLP-34/NPR-11 system is required for learning negative associations, a process that is reminiscent of NPY signaling in mammals. The Caenorhabditis elegans NPY/NPF ortholog FLP-34 displays conserved structural hallmarks of bilaterian-wide NPY/NPF neuropeptides. We show that it is required for aversive olfactory learning after pairing diacetyl with the absence of food, but not for appetitive olfactory learning in response to butanone. To mediate diacetyl learning and thus integrate the aversive food context with the diacetyl odor, FLP-34 is released from serotonergic neurons and signals through its evolutionarily conserved NPY/NPF GPCR, NPR-11, in downstream AIA interneurons. NPR-11 activation in the AIA integration center results in avoidance of a previously attractive stimulus. This study opens perspectives for a deeper understanding of stress conditions in which aversive learning results in excessive avoidance.SIGNIFICANCE STATEMENT Aversive learning evolved early in evolution to promote avoidance of dangerous and stressful situations. In addition to classical neurotransmitters, neuropeptides are emerging as modulators of complex behaviors, including learning and memory. Here, we identified the evolutionary ortholog of neuropeptide Y neuropeptide F in the nematode Caenorhabditis elegans, and we discovered that it is required for olfactory aversive learning. In addition, we elucidated the neural circuit underlying this avoidance behavior, and we discovered a novel coordinated action of Caenorhabditis elegans neuropeptide Y/neuropeptide F and serotonin that could aid in our understanding of the molecular mechanisms underlying stress disorders in which excessive avoidance results in maladaptive behaviors.

Disciplines
Medicine and Health Sciences

Publication Details

Authors
Melissa Fadda, Nathan De Fruyt, Charline Borghgraef, Jan Watteyne, Katleen Peymen, Elke Vandeweyer, Francisco Naranjo Galindo, Amanda Kieswetter, Olivier Mirabeau, Yee Lian Chew, Isabel Beets, and Liliane Schoofs

This journal article is available at Research Online: https://ro.uow.edu.au/ihmri/1543
Behavioral/Cognitive

NPY/NPF-Related Neuropeptide FLP-34 Signals from Serotonergic Neurons to Modulate Aversive Olfactory Learning in Caenorhabditis elegans

Melissa Fadda,1 Nathan De Fruyt,1 Charline Borghgraef,1 Jan Watteyne,1 Katleen Peymen,1 Elke Vandeweyer,1 Francisco J. Naranjo Galindo,1 Amanda Kieswetter,1 Olivier Mirabeau,2 Yee Lian Chew,3 Isabel Beets,1† and Liliane Schoofs1†

1Department of Biology, KU Leuven, Leuven, 3000, Belgium, 2Genetics and Biology of Cancers Unit, Institut Curie, Institut National de la Santé et de la Recherche Médicale U830, Paris Sciences et Lettres Research University, Paris, 75005, France, and 3Illawarra Health & Medical Research Institute School of Chemistry & Molecular Bioscience, University of Wollongong, Wollongong, 2522 New South Wales, Australia

Aversive learning is fundamental for animals to increase chances of survival. In addition to classical neurotransmitters, neuropeptides have emerged to modulate such complex behaviors. Among them, neuropeptide Y (NPY) is well known to promote aversive memory acquisition in mammals. Here we identify an NPY/neuropeptide F (NPF)-related neuropeptide system in Caenorhabditis elegans and show that this FLP-34/NPR-11 system is required for learning negative associations, a process that is reminiscent of NPY signaling in mammals. The Caenorhabditis elegans NPY/NPF ortholog FLP-34 displays conserved structural hallmarks of bilaterian-wide NPY/NPF neuropeptides. We show that it is required for aversive olfactory learning after pairing diacetyl with the absence of food, but not for appetitive olfactory learning in response to butanone. To mediate diacetyl learning and thus integrate the aversive food context with the diacetyl odor, FLP-34 is released from serotonergic neurons and signals through its evolutionarily conserved NPY/NPF GPCR, NPR-11, in downstream AIA interneurons. NPR-11 activation in the AIA integration center results in avoidance of a previously attractive stimulus. This study opens perspectives for a deeper understanding of stress conditions in which aversive learning results in excessive avoidance.

Key words: aversive learning; Caenorhabditis elegans; GPCR; neuropeptide Y; serotonin

Significance Statement

Aversive learning evolved early in evolution to promote avoidance of dangerous and stressful situations. In addition to classical neurotransmitters, neuropeptides are emerging as modulators of complex behaviors, including learning and memory. Here, we identified the evolutionary ortholog of neuropeptide Y/neuropeptide F in the nematode Caenorhabditis elegans, and we discovered that it is required for olfactory aversive learning. In addition, we elucidated the neural circuit underlying this avoidance behavior, and we discovered a novel coordinated action of Caenorhabditis elegans neuropeptide Y/neuropeptide F and serotonin that could aid in our understanding of the molecular mechanisms underlying stress disorders in which excessive avoidance results in maladaptive behaviors.

Received Nov. 12, 2019; revised Apr. 26, 2020; accepted June 12, 2020.


†I.B. and L.S. contributed equally to this work.

The authors declare no competing financial interests.

This work was supported by European Research Council Grant 340318 and the Research Foundation—Flanders Grant G062618N. The Caenorhabditis Genetics Center, funded by National Institutes of Health Office of Research Infrastructure Programs P40 OD010440, for providing the Caenorhabditis elegans strains used in this study. We thank Cori Bargmann for providing the pSM vector, the CX8912 and CX7894 strains, and advice on the manuscript; and Gunther Hoppel for providing the pG4H96-Cas9 expressing vector.

Correspondence should be addressed to Liliane Schoofs at liliane.schoofs@kuleuven.be or Isabel Beets at isabel.beets@kuleuven.be.

https://doi.org/10.1523/JNEUROSCI.2674-19.2020

Copyright © 2020 the authors

Introduction

Learning and memory processes evolved early in evolution to benefit survival of animals in a dynamic environment. Fundamental insights into the molecular substrates of learning and memory have first been described in the mollusc Aplysia californica (Pinsker et al., 1970; Frost et al., 1985). Subsequent studies in vertebrate and invertebrate models revealed the conservation of these molecular principles across animal phyla (Ardiel and Rankin, 2010; Kandel, 2012).

Caenorhabditis elegans has proven to be a powerful model for dissecting molecular mechanisms involved in experience-dependent plasticity (Hobert, 2003). The C. elegans nervous system is compact and displays a broad range of experience-dependent
behaviors, such as nonassociative and associative learning (Ardiel and Rankin, 2010; Sasakura and Mori, 2013). Most classical conditioning paradigms rely on pairing aversive or favorable feeding states with gustatory, thermal, and odorant cues to shape the animals’ innate attraction or repulsion toward these stimuli, leading to experience-dependent remodeling of neural circuits (Stetak et al., 2009; Ardiel and Rankin, 2010). In addition to classic neurotransmitter systems, the C. elegans genome encodes a large number of neuropeptide precursor proteins that are predicted to generate over 300 bioactive peptides (Van Bael et al., 2018a). These evolutionarily ancient molecules mainly act through GPCRs, most of which are also conserved across animal phyla (Jékely, 2013; Mirabeau and Joly, 2013; Elphick et al., 2018). The structural diversity of neuropeptidergic systems underlies a broad range of physiological and behavioral functions, including learning and memory (Beets et al., 2012; Taghert and Nitabach, 2012; Peymen et al., 2019).

One highly conserved neuropeptidergic system involved in learning and memory in both vertebrates and invertebrates is the neuropeptide Y/neuropeptide F (NPY/NPF) system (Krashes et al., 2009; Gotzsche and Woldbye, 2016). Vertebrate NPY and its vertebrate NPF, as well as their receptors (NPYRs/NPFRs), were found in nearly all bilaterian phyla investigated to date (Fadda et al., 2019). In mammals, NPY is abundantly expressed in the CNS where it has varying effects on learning and memory formation depending on the brain region, the type of memory, and the NPYR subtypes activated (Gotzsche and Woldbye, 2016). NPYRs are Gs,Gt-coupled receptors that are mainly postsynaptically located on glutamatergic neurons of the limbic system, where they exert antieccitatory actions by decreasing glutamate release (Vollmer et al., 2016). In addition to a role in learning and memory formation, disruption of NPY signaling has been shown to decrease serotonin levels, leading to behavioral inflexibility and territorial aggressive behavior (Karl et al., 2004). In Drosophila, the NPY ortholog NPFR regulates appetitive learning in accordance with feeding state (Krashes et al., 2009). Food deprivation induces the release of NPF, which promotes appetitive olfactory learning by inhibiting dopaminergic neurons that project to the mushroom body (Krashes et al., 2009), an important integration center in flies.

In C. elegans, several neuropeptide receptors (NPRs) have been predicted as putative NPY/NPF-like receptor orthologs based on sequence similarities (Keating et al., 2003). A large-scale phylogenetic analysis of neuropeptide systems across bilaterian animals revealed NPR-11 to be the closest C. elegans ortholog of the NPY/NPF receptor family (Mirabeau and Joly, 2013). This receptor has been shown to modulate local search behavior and chemotaxis (CTX) in response to the attractive odor isoamyl alcohol, sensed by AWC neurons. NPR-11 acts in AIA interneurons postsynaptic to AWC and its activation reduces AWC responses to isoamyl alcohol, a feedback mechanism that promotes odor adaptation (Chalasani et al., 2010). Given that NPF signaling is known to regulate learning in mammals (Gotzsche and Woldbye, 2016) and appetitive memory in Drosophila (Krashes et al., 2009), we hypothesized that the NPY/NPFR ortholog NPR-11 is involved in food-dependent learning and memory in C. elegans. Here, we demonstrate that npr-11 is not required for appetitive olfactory learning but regulates aversive learning in response to the odor diacetyl. NPY/NPF-related neuropeptides, encoded by ftp-34, signal from serotonergic neurons through NPR-11 in AIA interneurons of the olfactory circuit to mediate aversive learning.
Control worms for this experiment, showing desensitization, were conditioned for 90 min on CTX plates with 2 µl of undiluted diacetyl pipetted on the lid.

Next-generation of transgenic worms. The cell-specific rescue construct for flp-34 was cloned using NEBuilder HiFi DNA Assembly (NEB) in a pSM vector (kindly provided by C. Bargmann, Rockefeller University). The cDNA of flp-34 was amplified by PCR and cloned at the KpnI site. The tph-1 promoter sequence (428 bp) includes a premature stop codon at the beginning of exon 2 (signal peptide region), to prevent further translation of the mature peptide encoded by flp-34. The locomotion activity of 15-20 young adult worms was recorded in well-fed condition (on 250 μl of NGM plates) or after 1 h of food deprivation (on NGM-unseeded plates).

The plasmid for heterologous expression of npr-11 in CHO cells was obtained by directionally cloning the PCR-amplified npr-11 cDNA into the pcDNA3.1/V5-His-TOPO TA vector (Invitrogen). Two guide RNA (gRNA) sequences were designed (https://eu.idtdna.com/site/order/designtool/index/CRISPR_SEQUENCE) as well as a repair sequence (as previously reported (Arribere et al., 2014; Paix et al., 2015). A mix containing all the required components was injected into C. elegans young adult hermaphrodites (Table 3).

The plasmid for heterologous expression of npr-11 in CHO cells was obtained by directionally cloning the PCR-amplified npr-11 cDNA into the pcDNA3.1/V5-His-TOPO TA vector (Invitrogen). Two guide RNA (gRNA) sequences were designed (https://eu.idtdna.com/site/order/designtool/index/CRISPR_SEQUENCE) as well as a repair sequence (as previously reported (Arribere et al., 2014; Paix et al., 2015). A mix containing all the required components was injected into C. elegans young adult hermaphrodites (Table 3).

The plasmid for heterologous expression of npr-11 in CHO cells was obtained by directionally cloning the PCR-amplified npr-11 cDNA into the pcDNA3.1/V5-His-TOPO TA vector (Invitrogen). Two guide RNA (gRNA) sequences were designed (https://eu.idtdna.com/site/order/designtool/index/CRISPR_SEQUENCE) as well as a repair sequence (as previously reported (Arribere et al., 2014; Paix et al., 2015). A mix containing all the required components was injected into C. elegans young adult hermaphrodites (Table 3).

The plasmid for heterologous expression of npr-11 in CHO cells was obtained by directionally cloning the PCR-amplified npr-11 cDNA into the pcDNA3.1/V5-His-TOPO TA vector (Invitrogen). Two guide RNA (gRNA) sequences were designed (https://eu.idtdna.com/site/order/designtool/index/CRISPR_SEQUENCE) as well as a repair sequence (as previously reported (Arribere et al., 2014; Paix et al., 2015). A mix containing all the required components was injected into C. elegans young adult hermaphrodites (Table 3).
perpendicularly on the glass stage where the assay plates were positioned to enhance the contrast. The StreamPix Multicamera 6 software was used to record and acquire video streams that were successively converted into frames. A custom MATLAB R2016a (MathWorks) script was used to track individual worms over consecutive frames. Each track was then visually evaluated to discard immobile background artifacts. The average speed of the worms along the recorded 10 min was obtained as output of the analysis and used to compare the locomotion activity of different worm strains. The custom MATLAB script is available on request.

Expression pattern analysis. Hermaphrodite transgenic worms were mounted on a 2% agarose pad and immobilized with 1 mM Na3 (Sigma Millipore) in M9 buffer. The expression pattern of the transgenic animals was visualized using an Olympus Fluoview FV1000 (IX81) confocal microscope, and the z-stack projections were analyzed with Imaris 7.2 (Bitplane) software. For fisd-mid-based npr-1::ss2::gfp and flp-34::ss2::gfp transgenes, cell identifications were based on the following position and morphology, colocalization with Dil (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, Invitrogen) staining (Tong and Bürglin, 2010) and crossing with marker strains (tpel-1::DsRed2, nlp-4::mCherry, and srxs-3::gfp;str-2::dsRed2, kindly provided by C. Danote) and the respective repair template sequences (Table 4).

Receptor activation assay. The in vitro GPCR activation assay for NPR-11 was performed using an acquirin-based luminescence assay as reported previously (Beets et al., 2012; Van Sinay et al., 2017; Peymen et al., 2019). Briefly, CHO cells stably expressing the acquirin calcium indicator and the promiscuous Ga16 protein (ES-000-A24, PerkinElmer) were transfected with pcDNA3.1/npr-1 or pcDNA3.1 empty vector at 40%–50% confluency using Lipofectamine LTX and Plus Reagent (Invitrogen). After 24 h at 37°C, the cells were moved to 28°C O/N. On the day of the screening, cells were collected at a density of 5 × 10^6 cells/ml in DMEM/F12 medium without phenol red (Invitrogen) supplemented with 0.1% BSA and loaded with coelenterazine H (Invitrogen) to reconstitute the Ca2+-sensitive photoprotein acquirin. Compton plates containing a library of over 350 peptides of the RFamide and neuropeptide-like protein (NLP) family. The library was compiled based on in silico predictions and peptidomics data (Husson et al., 2005; Van Bael et al., 2018b), and was custom-synthesized by Thermofisher Scientific and GL Biochem. FLP-34 and NLP-1 peptides used for dose–response experiments were purified by reversed-phase high-performance liquid chromatography on a Symmetry-C18 column (4.6 × 250 mm HPLC cartridge with pore size of 5 μm) and quantified with the bicinchoninic acid protein assay. The mass of peptides was verified by MALDI-TOF mass spectrometry (matrix assisted laser desorption/ionization time-of-flight analyzer) on a Reflex IV instrument (Bruker Daltonics).

Experimental design and statistical analysis. The exon-intron structure of genes encoding NPF/NPY peptides. The NPF/NPY alignment was performed using ClustalX 2.1 Multiple Sequence Alignment with default parameters (Jeanmougin et al., 1998; Larkin et al., 2007). The source of the bilaterian representative NPF/NPY mature peptides is indicated in the Table 4, together with species name abbreviations. The software BOXSHADE (www.ch.embnet.org/software/BOX_form.html) was used to highlight conserved amino acids: identical residues with a minimum of 70% conservation are highlighted in black, amino acid groups with strongly similar properties and a minimum of 70% conservation are highlighted in dark gray, and amino acid groups with strongly similar properties and a minimum of 55% conservation are highlighted in light gray.

Analysis of the exon-intron structure of genes encoding NPF/NPY was performed by comparing nucleotides, transcripts, and precursor proteins encoding for representative NPF/NPY peptides across bilaterans. Nucleotide and amino acid sequences were obtained from the NCBI, WormBase, and the Joint Genome Institute databases. Accession numbers of the sequences used for the analysis, together with species name abbreviations, are listed in the Table 5.
the statistical significance between experimental groups and the total number of replicates (indicated by \( n \) for learning assays and receptor activation assays) or worms (indicated by \( w \) for locomotion assays) for each experiment are reported in the figure legends.

Learning assays were performed on at least two independent days, including each time two to four replicates per condition for the diacetyl learning assays and 5 replicates for the butanone-positive association assay. For diacetyl learning assays, comparing different conditions for one genotype, data were analyzed using a Kruskal-Wallis test and Dunn’s post hoc test for multiple comparison (nonparametric data) or a one-way ANOVA and Tukey’s multiple comparisons test (for parametric data). For diacetyl learning and butanone learning assays comparing different conditions for multiple genotypes, data were analyzed using two-way ANOVA and Sidak’s or Tukey’s multiple comparison tests. Locomotion assays were performed in triplicate on 2 independent days, and the difference in average speed was analyzed with an unpaired \( t \) test. Receptor activation assays for dose–response measurements were done in triplicate on at least 2 independent days. The comparison between calcium responses of FLP-34 and NLP-1 peptides in NPR-11-expressing cells was performed in duplicate in three independent experiments. Data were analyzed using Kruskal–Wallis test and Dunn’s post hoc test.

### Results

**Npr-11 is not required for appetitive olfactory learning in response to butanone**

In *Drosophila melanogaster*, RNAi knockdown of the *npfr* gene impairs appetitive olfactory learning (Krashes et al., 2009). In *C. elegans*, the ortholog of this receptor, encoded by the *npr-11* gene (Mirabeau and Jolly, 2013), mediates adaptation to the attractive odor isooamyl alcohol, sensed by olfactory AWC neurons (Chalasani et al., 2010). Based on these findings, we hypothesized that NPR-11 is involved in AWC-mediated appetitive learning. To test this, we quantified the performance of *npr-11* mutants in an established appetitive learning assay referred to as positive butanone association in which worms learn to associate food with butanone, an odorant sensed by AWC (Kauffman et al., 2011). We exposed the worms to food deprivation for 1 h, conditioned them for the same amount of time with butanone in the presence of food, and quantified CTX behavior to butanone before and after conditioning by calculating a CI (Fig. 1A). Untrained WT *C. elegans* were moderately attracted to butanone (Fig. 1B). As expected, conditioned worms showed a strongly increased CI and thus enhanced attraction to the odorant immediately after conditioning (time 0, t0). This increased attraction to butanone gradually declined over 2 h when animals were kept on food in the absence of butanone (Fig. 1B). Since the naive attraction of WT animals to butanone was slightly lower compared with CI values reported previously (Kauffman et al., 2011), we validated the identities of AWC neurons in this WT strain by investigating the asymmetric expression of *str-2*, encoding a receptor that senses the butanone odour (Troemel et al., 1999).

### Npr-11 mutants are defective in aversive olfactory learning

Since NPR-11 signaling is not required for appetitive olfactory learning in response to butanone, we asked whether it is involved in learning negative associations, a process that is reminiscent of NPY signaling in mammals (Gotzsche and Woldbye, 2016). To investigate whether *npr-11* mutants are defective in aversive olfactory learning, we modified an established conditioning protocol (Stetak et al., 2009; Vukojevic et al., 2012) pairing short-term food deprivation with exposure to diluted diacetyl, which we refer to as diacetyl learning (Fig. 2A). After pairing diacetyl with the absence of food, the behavioral preference of worms toward the odorant diacetyl is switched from attractive to repulsive.

In our modified protocol, we conditioned animals with 0.1% diluted diacetyl for 3 h in the absence of food. We first tested whether this training elicits aversive associative learning. Before conditioning, WT worms were strongly attracted toward diacetyl and showed a highly positive CI (Fig. 2B). As expected for aversive learning, after pairing food deprivation with exposure to diacetyl, attraction toward the odorant was reduced, resulting in a significantly decreased CI (Fig. 2B).
paired association of diacetyl and the absence of food, and not from exposure to one of the stimuli, we tested two unpaired controls in which animals were exposed only to the diacetyl in the presence of food or only to food deprivation without diacetyl. WT worms conditioned with both diacetyl and the absence of food were less attracted toward diacetyl. However, their CI for naive chemotaxis (1h) = 10% butanone − no. (no butanone) no. (tot) − no. (origin).

**Legend**
- no butanone
- 10% butanone

**Chemotaxis index**

<table>
<thead>
<tr>
<th>Condition</th>
<th>naive chemotaxis (1h)</th>
<th>learning chemotaxis (1h)</th>
<th>memory chemotaxis (1h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>no butanone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% butanone</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**B**

![Chart](chart.png)

**C**

*kyIs408* x wild type (4x outcrossed)

**Legend**
- wild type
- *npr-11*(ok594)

**Figure 1.** *npr-11* mutants show normal appetitive olfactory learning in response to butanone. A, Schematic of the positive butanone association paradigm (Kauffman et al., 2011). Age-synchronized worms are washed from the cultivation plates and immediately tested for naive CTX to butanone. The remaining animals undergo 1 h of food deprivation followed by 1 h of conditioning with food and butanone. A part of the population is immediately tested for CTX to butanone (learning CTX, t0), while the rest of the animals are kept on food-seeded plates for 30, 60, or 120 min before testing CTX to butanone (memory CTX). B, *npr-11* mutants are not defective in positive butanone association. For each time point (t0 = 0, t30 = 30, t60 = 60, and t120 = 120 min after conditioning), the CI values of *npr-11* mutants are similar to those of WT. Data were analyzed by two-way ANOVA (*F*<sub>4,90</sub> = 10.25, *p* < 0.0001) and Sidak’s multiple comparison test (*n* = 10). Boxplots represent 25th (lower boundary) and 75th (upper boundary) percentiles. The 50th percentile (line) shows the median. Whiskers plot the minimum and maximum values. Black dots represent individual CIs. C, WT animals show asymmetric expression of str-2 and *srsx-3* receptor-encoding genes in AWCON and AWCOFF neurons. Representative confocal z-stack projections of AWCON neurons in transgenic adult hermaphrodites expressing a *srsx-3::gfp; str-2::dsRed2* transgene (*kyIs408*) in a reporter strain (CJ794, kindly provided by C. Bargmann, Rockefeller University) before (left) and after (right) crossing with the WT strain used in positive butanone association assays. A, Anterior; P, posterior; D, dorsal; V, ventral. Scale bars, 10 μm. ns, not significant.

mechanical and thermal shock treatment that erases short-term memory, but not desensitization (Nuttley et al., 2001). As a control for desensitization, we included an experimental group exposed to undiluted diacetyl for 90 min (Colbert and Bargmann, 1997). Animals conditioned with 0.1% diacetyl showed a significant increase of CI after shock treatment, as expected for learning (Fig. 2D). The same treatment did not affect the CI of the control group exposed to undiluted diacetyl, characteristic of desensitization (Fig. 2D). Together, these results indicate that 3 h exposure to 0.1% diacetyl in the absence of food elicits aversive associative learning.

We next evaluated diacetyl learning of *npr-11* loss-of-function mutants. In contrast to WT animals, *npr-11* mutants were still strongly attracted toward diacetyl after conditioning (Fig. 2E). Since this diacetyl learning defect could be caused by a general defect in neural circuits involved in locomotion, we quantified the speed of *npr-11* mutants in well-fed conditions and after 1 h of food deprivation, to test potential locomotory defects. Mutant animals moved at an average speed similar to that of WT worms in both fed and starved conditions (Fig. 2F,G). Untrained *npr-11* mutants also showed normal attraction to diacetyl (Fig. 2E). Together, these results suggest that *npr-11* signaling does not affect general locomotion or diacetyl sensing but is required for diacetyl learning.
Figure 2.  

**A.** Schematic of the diacetyl learning assay, modified from Vukojevic et al., (2012). Age-synchronized worms are washed from cultivation plates and immediately tested for naive CTX to diacetyl, while the remaining animals undergo 3 h of coupled (diacetyl + no food) or uncoupled (diacetyl-only or no food-only) conditioning. CTX to diacetyl is then quantified on CTX plates; and after 1 h, a CI is calculated.  

**B.** WT worms after coupled conditioning (cond.) show a significant drop in the CI compared with animals conditioned with diacetyl-only (left) or no food-only (right). Data were analyzed by Kruskal–Wallis (KW(2) = 24.15, \( p < 0.0001 \)) and Dunn’s multiple comparison tests (left, \( n = 21 \)) or one-way ANOVA (\( F(2,13) = 17.37, \ p = 0.0002 \)) and Tukey’s post hoc test (right, \( n = 5 \)).  

**C.** nmr-1 mutants show impaired diacetyl learning compared with WT. Two-way ANOVA revealed a significant effect of genotypes (\( F(1,30) = 13.83, \ p = 0.0008 \)), of behavioral treatments (\( F(1,30) = 83.83, \ p < 0.0001 \)), and of interaction between genotypes and behavioral treatments (\( F(1,30) = 13.14, \ p = 0.0011 \)). Data were analyzed by Sidak’s multiple comparison test (\( n = 7 \)).  

**D.** Shock treatment reverses the reduced attraction to diacetyl in animals conditioned for diacetyl learning (cond.), but not in a control group for desensitization exposed to undiluted diacetyl. Data were analyzed by one-way ANOVA (\( F(7,33) = 11.50, \ p = 0.0001 \)) and Tukey’s post hoc test (\( n = 4 \)).  

**E.** npr-11 mutants show normal attraction to diacetyl before conditioning but are defective in diacetyl learning. Two-way ANOVA revealed a significant effect of genotypes (\( F(1,46) = 7.66, \ p = 0.0081 \)), of behavioral treatments (\( F(1,46) = 94.92, \ p < 0.0001 \)), and of interaction between genotypes and behavioral treatments (\( F(1,46) = 14.61, \ p = 0.0004 \)). Data were analyzed by Sidak’s post hoc test (\( n = 10 \)).  

**F, G.** npr-11 mutants display normal locomotion speed on and off food. Activity is recorded for 10 min on E. coli OP50-seeded (F) or unseeded (G) NGM plates. The average speed of mutants is not significantly different from that of WT in both conditions. Data were analyzed by unpaired t test (\( F, \ w \geq 23; \ G, \ w \geq 39 \)). B–G, Boxplots represent 25th (lower boundary) and 75th (upper boundary) percentiles. The 50th percentile (line) indicates the median. Whiskers represent the minimum and maximum values. Black dots represent individual CIs or worms. *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \), ****\( p < 0.0001 \). ns, not significant.
NPR-11 is required in AIA interneurons for diacetyl learning

To shed light on the cells in which NPR-11 is required for diacetyl learning, we examined the expression pattern of the receptor. Worms expressing a fosmid-recombineered GFP transgene for \( npr-11 \) showed fluorescence in several head and tail neurons (Fig. 3A). This transgene confirmed the previously reported expression of \( npr-11 \) in AIA interneurons (Chalasani et al., 2010) (Fig. 3A). In addition, we observed \( npr-11 \) expression in ASK and ADL sensory neurons, validated by Dil staining of amphid neurons, as well as in several unidentified cells in the head (Fig. 3A). In the tail, the \( npr-11 \) reporter transgene was expressed in PVQ and most likely in DVA or DVC neurons (Fig. 3A).

AIA interneurons play a well-described role as sensory integration centers involved in aversive learning (Chalasani et al., 2010; Dobosiewicz et al., 2019). We therefore asked whether \( npr-11 \) is required in these neurons for diacetyl learning. Restoring \( npr-11 \) expression in AIA, under control of the \( gcy-28.d \) promoter, was sufficient to rescue diacetyl learning of \( npr-11 \) mutants to the level of WT worms, indicating that NPR-11 acts in AIA to mediate this type of learning (Fig. 3B).
**Nlp-1 mutants display normal diacetyl learning**

One of the buccalin-related NLP-1 neuropeptides (MDANAFRMSFa) has been reported to activate NPR-11 and thereby inhibit local search behavior (Chalasani et al., 2010). Since npr-11 is also required for diacetyl learning, we tested whether this effect is mediated by NLP-1. If NLP-1 neuropeptides modulate diacetyl learning by activating NPR-11, we expect nlp-1 mutants to recapitulate the learning defect observed for npr-11 in the diacetyl assay. However, after 3 h of conditioning with diacetyl in the absence of food, nlp-1 mutants showed a reduced attraction to diacetyl similar to the behavior of WT animals (Fig. 3C). These results suggest that NLP-1 is not required for diacetyl learning and NPR-11 may be activated by other ligands in the aversive olfactory learning circuit.

**The C. elegans NPY/NPF ortholog FLP-34 dose-dependently activates NPR-11 in vitro**

To search for neuropeptide ligands of NPR-11 involved in diacetyl learning, we used a reverse pharmacology approach and tested the functional response of this GPCR to a synthetic library of C. elegans peptides in a calcium-based reporter assay (Fig. 4A). In this cellular assay, Chinese Hamster Ovary (CHO) cells expressing NPR-11 were challenged with a library of over 350 FLP and NLP peptides. Two peptides derived from the flp-34 neuropeptide precursor gene dose-dependently activated NPR-11 with EC50 values in the nanomolar range (neuropeptide precursor gene dose-dependently activated FLP and NLP peptides. Two peptides derived from the expressing NPR-11 were expected to recapitulate the learning defect observed for npr-11 in the diacetyl assay. However, after 3 h of conditioning with diacetyl in the absence of food, nlp-1 mutants showed a reduced attraction to diacetyl similar to the behavior of WT animals (Fig. 3C). These results suggest that NLP-1 is not required for diacetyl learning and NPR-11 may be activated by other ligands in the aversive olfactory learning circuit.

**Loss-of-function mutants of flp-34 phenocopy the diacetyl learning defect of npr-11 mutants**

Because FLP-34 neuropeptides activate NPR-11 in vitro, we asked whether, like NPR-11, they are involved in diacetyl learning. Thus, flp-34 mutants should display the same learning defect as npr-11 mutants. As no null mutants for flp-34 were available, we used CRISPR/Cas9 genome editing to generate a flp-34 loss-of-function mutant. For this, we designed two gRNAs to delete exon 2 and a major part of exon 3. In addition, the repair template recoded a premature stop codon in the signal peptide region to prevent further translation of the remaining sequence, including both FLP-34 peptides (Fig. 6A, Extended Data Fig. 6-1). Like npr-11 mutants, mutants defective in flp-34 still showed strong attraction to diacetyl after training (Fig. 6B), whereas locomotion behavior was unaffected (Fig. 6C,D). Moreover, a double mutant of npr-11 and flp-34 displayed a learning defect similar to that of single npr-11 and flp-34 mutants (Fig. 6B). The absence of an additive effect in the double mutant indicates that flp-34 and npr-11 act in the same genetic pathway. Together, these results suggest that FLP-34 neuropeptides signal through NPR-11 in vivo to mediate diacetyl learning.

**Flp-34 expression in serotonergic neurons is required for diacetyl learning**

Next, we asked which cell(s) express flp-34 to understand further the NPY/NPF neural circuit modulating diacetyl learning. We generated a fosmid-based reporter transgene for flp-34 and found consistent fluorescence in several head and tail neurons, as well as in the vulval region (Fig. 7A). The flp-34 reporter transgene was expressed in all serotonergic neurons (NSM, HSN, and ADF) (Horvitz et al., 1982), validated by colocalization of the GFP signal with the expression of a red reporter transgene for tryptophan hydroxylase 1 (tph-1::DsRed2) that encodes a serotonin biosynthesis enzyme (Sze et al., 2002) (Fig. 7A). The flp-34 transgene was also expressed in the ASG sensory neurons as confirmed by colocalization with an ASG specific reporter (nlp-44:: mCherry), in the tail PHA sensory neurons as confirmed by Dil staining, and in the PLN and ALN neurons as identified by their morphology and position (Fig. 7A).

Given the correlation between serotonin signaling and feeding state in C. elegans (Srinivasan et al., 2008), we hypothesized that NPY/NPF-like neuropeptides signal from these serotonergic cells to mediate diacetyl learning. We cell-specifically restored flp-34 expression in NSM, HSN, and ADF neurons using the tph-1 promoter (Sze et al., 2002), which rescued the diacetyl learning defect (Fig. 7B). By contrast, restoring flp-34 in other neurons that express the flp-34 gene, as evidenced by reporter transgene expression (Fig. 7A) or single-cell RNA-sequencing (Cao et al., 2017; Taylor et al., 2019), did not rescue this mutant phenotype. Restoring expression of flp-34 in ASK/ASI, ASG, or PHA neurons did not restore the learning defect of flp-34 mutants (Fig. 7B). This suggests that flp-34 is specifically required in serotonergic cells to facilitate diacetyl learning rather than that learning is mediated by unregulated neuropeptide secretion. Together, these data show that NPY/NPF-related neuropeptides signal from serotonergic neurons, and possibly other unidentified cells, to NPR-11 in AIA interneurons regulating diacetyl learning.

**NPY/NPF-like FLP-34 neuropeptides and serotonin act in the same pathway to mediate diacetyl learning**

The requirement of flp-34 in serotonergic neurons for diacetyl learning suggests that NPY/NPF-like neuropeptides and serotonin act together to mediate aversive learning. To investigate this, we tested diacetyl learning for tph-1 mutants, defective in serotonin biosynthesis. After conditioning, tph-1 mutants displayed a learning defect, which was more severe than that of npr-11 mutants (Fig. 8A). This suggests that serotonin, in addition to NPY/NPF-like signaling, mediates diacetyl learning. We next generated a tph-1::npr-11 double mutant to simultaneously block serotonin and NPYR/NPFR signaling, and compared diacetyl learning of the single and double mutants. The learning defect displayed by tph-1::npr-11 mutants was not significantly different from that of tph-1 mutant animals (Fig. 8A), suggesting that serotonin and NPR-11 act in the same pathway to regulate diacetyl learning (Fig. 8B).
Discussion
Several molecular mechanisms for learning and memory have shown to be conserved during evolution (Glanzman, 2008; Ardiel and Rankin, 2010; Kandel, 2012). Mounting evidence implies neuropeptides to be key modulators of experience-dependent behaviors (Bargmann, 2012). Here, we show that NPY/NPF-related neuropeptides from serotonergic neurons signal through the NPYR/NPFR ortholog NPR-11 to mediate aversive olfactory learning in C. elegans.
Several bioinformatic studies have classified C. elegans NPRs as putative NPYR/NPFR orthologs (Keating et al., 2003). NPR-1 was first annotated as an NPYR homolog based on its sequence similarity with human NPYRs (de Bono and Bargmann, 1998). Later on, 11 additional C. elegans NPRs (NPR-2 to −8 and NPR-10 to −13) were predicted as NPYR/NPFR homologs based on a phylogenetic analysis of C. elegans GPCRs (Keating et al., 2003). However, bilaterian-wide phylogenetic analyses of neuropeptide GPCRs revealed that the majority of these putative NPYR/NPFRs in C. elegans cluster closely with insect short NPF receptors.
Figure 6. *fhp-34* mutants phenocopy the defect of *npr-11* mutants in diacetyl learning. A, CRISPR/Cas9-induced deletion in a *fhp-34* loss-of-function mutant. Boxes represent exons encoding *fhp-34*, numbered 1-5. Lines between boxes indicate introns. Part of the intron between exons 1 and 2 is cropped. Red represents the CRISPR/Cas9 deletion (*lst1666*). A stop codon introduced in exon 2 interrupts translation of the signal peptide region, preventing the synthesis of downstream sequences containing both mature peptides. Horizontal lines indicate signal peptide (black) and mature peptide sequences (green). Additional information about the CRISPR/Cas9-edited deletion can be found in Extended Data Figure 6-1. B, *fhp-34* mutants are defective in diacetyl learning. The CI of *fhp-34* mutants after conditioning is similar to that of conditioned *npr-11* mutant animals and significantly different from WT. An *npr-11;fhp-34* double mutant does not display additive learning defects. Two-way ANOVA revealed a significant effect of genotypes (*F*<sub>(2,32) = 3.262, *p* = 0.0286), of behavioral treatments (*F*<sub>(3,32) = 116.1, *p* < 0.0001), and of interaction between genotypes and behavioral treatments (*F*<sub>(6,96) = 4.048, *p* = 0.0116). Data were analyzed by Sidak’s post hoc test (n ≥ 7). C, D, *fhp-34* mutants display normal locomotion speed on and off food. Activity is recorded for 10 min on OP50-seeded (C) or unseeded (D) NGM plates. The average speed of mutants is not significantly different from that of WT in both conditions. Data were analyzed by unpaired t test (C, w ≥ 75; D, w ≥ 11). B-D, Boxplots represent 25th (lower boundary) and 75th (upper boundary) percentiles. The 50th percentile (line) indicates the median. Whiskers represent the minimum and maximum values. Black dots represent individual CI5s or worms. **p < 0.01, ***p < 0.001, ****p < 0.0001. ns, not significant.

sugesting that this family has largely expanded in nematodes (Jékely, 2013; Mirabeau and Joly, 2013). These studies also revealed NPR-11 as the genuine *C. elegans* ortholog of the bilaterian-wide NPYR/NPFR family (Jékely, 2013; Mirabeau and Joly, 2013). A recent study showed that pharmacological concentrations of human NPY activate *C. elegans* NPR-11 in *vivo*, whereas the same or higher concentrations of human NPY (10 μM) elicited weak or no responses by other tested *C. elegans* NPRs (Gershkovich et al., 2019).

Here we show that the NPY/NPF-like FLP-34 neuropeptides are cognate ligands of NPR-11. Several of our results support the orthology of these neuropeptides with the bilaterian NPY/NPF family: (1) the FLP-34 neuropeptide precursor contains two peptides, FLP-34-1 and FLP-34-2, which display structural hallmarks of bilaterian NPY/NPF neuropeptides. These include the evolutionarily conserved C-terminal RXRF/Yamide motif, which is distinct from the M/T/L/FRFamide motif of sNPY neuropeptides (Fadda et al., 2019). (2) Both FLP-34-1 and FLP-34-2 peptides dose-dependently activate the NPYR/NPFR ortholog NPR-11 in *vivo* with EC<sub>50</sub> values in the nanomolar range. (3) The *fhp-34* gene also displays a conserved exon-intron junction that is retained in the same identical position in most of the analyzed bilaterian *npf/npy* genes, including insect (*A. aegypti, B. mori,* and *D. pulex*), nematode (*P. pacificus*), mollusc (*L. gigantea*), annelid (*C. capitata*), and vertebrate (*D. rerio, G. gallus, M. musculus,* and *H. sapiens*) homologs. Remarkably, the conservation of this exon-intron structure is not retained in *Drosophila npf*. Although the FLP-34 precursor contains two mature NPY/NPF-like peptides, only the C-terminal sequence of FLP-34-2 is interrupted by the conserved exon-intron junction, suggesting that FLP-34-1 may be a more recent duplication of FLP-34-2 in nematodes. This is supported by the fact that NPY/NPF precursors outside nematodes produce only a single mature peptide, whereas in nematodes they contain two NPFs (McCoy et al., 2014).
Figure 7. **flp-34** is required in serotonergic neurons for diacetyl learning. A, Representative confocal z-stack projections of head, body, and tail neurons expressing a fosmid-based **flp-34::sl2::gfp** transgene in adult hermaphrodites. **flp-34** expression in the serotonergic neurons NSM, ADF (head panel), and HSN (body panel) was identified by colocalization with a tph-1p::DsRed2 marker transgene. Arrowheads indicate head and body projections from tail neurons. **flp-34** expression in ASG neurons (head panel) was identified by colocalization with nlp-44p::mCherry marker transgene. **flp-34** expression in the tail neuron PHA was identified by colocalization with DiI staining, whereas ALN and PLN were determined by morphology and position (tail panel). A, Anterior; P, posterior; D, dorsal; V, ventral. Scale bars, 10 μm. B, **flp-34** acts in serotonergic neurons to regulate diacetyl learning. Top left, Expressing WT copies of **flp-34** under control of the qdh-1 (NSM, HSN, ADF) promoter in serotonergic neurons partially rescues the learning defect of **flp-34** mutant animals. The expression of **flp-34** under control of ops-1 (ASG) (top right), gcy-27 (ASK, ASJ) (bottom left), and gcy-17 (PHA) (bottom right) promoters does not rescue the learning defect of **flp-34** mutants. (NSM, HSN, ADF) Two-way ANOVA revealed a significant effect of genotypes ($F_{(2,63)} = 5.333$, $p = 0.0072$), of behavioral treatments ($F_{(1,63)} = 267.4$, $p < 0.0001$), and of interaction between genotypes and behavioral treatments ($F_{(2,63)} = 11.28$, $p < 0.0001$). Data were analyzed by Sidak's and Tukey's post hoc test ($n = 21$). (ASG) Two-way ANOVA revealed a significant effect of genotypes ($F_{(2,40)} = 9.418$, $p = 0.0004$), of behavioral treatments ($F_{(1,40)} = 53.87$, $p < 0.0001$), and of interaction between genotypes and behavioral treatments ($F_{(2,40)} = 6.521$, $p = 0.0035$). Data were analyzed by Sidak's and Tukey's post hoc test ($n = 8$). (ASK, ASJ) Two-way ANOVA revealed a significant effect of genotypes ($F_{(2,66)} = 16.66$, $p < 0.0001$), of behavioral treatments ($F_{(1,66)} = 194.3$, $p < 0.0001$), and of interaction between genotypes and behavioral treatments ($F_{(2,66)} = 15.59$, $p < 0.0001$). Data were analyzed by Sidak's and Tukey's post hoc test ($n = 12$). (PHA) Two-way ANOVA revealed a significant effect of genotypes ($F_{(2,66)} = 16.77$, $p < 0.0001$), of behavioral treatments ($F_{(1,66)} = 161.6$, $p < 0.0001$), and of interaction between genotypes and behavioral treatments ($F_{(2,66)} = 17.90$, $p < 0.0001$). Data were...
also requires NPY signaling (Morley et al., 1990; Götzsche and Woldbye, 2016). In adult *Drosophila*, NPF signaling mediates appetitive learning under conditions of food deprivation and is thought to mimic the absence of food (Krashes et al., 2009). The absence of food might also trigger FLP-34 release from *C. elegans*...
serotonergic neurons in diacetyl learning. This is consistent with our findings that deficient NPYR/NPFR signaling in *C. elegans* does not affect appetitive learning in the positive butanone association paradigm, where worms are exposed to butanone in the presence of food.

How does FLP-34/NPR-11 signaling modulate aversive olfactory learning? Together with previous work, our results suggest the following model for diacetyl learning (Fig. 8B). The simultaneous presentation of diacetyl with food deprivation leads to the activation of neurons sensing odors and feeding state. Diacetyl is sensed by the ODR-10 receptor in AWA neurons (Sengupta et al., 1996), which in turn depolarize AIA interneurons via gap junctions (Larsch et al., 2015). The absence of food, on the other hand, is signaled by a three-neuron-type circuit in which octopamine triggers CREB-regulated gene expression in SIA neurons to initiate a behavioral response to food deprivation (Suo et al., 2009). In addition, serotonergic neurons are well known for their role in signaling internal and external feeding cues (Horvitz et al., 1982; Srinivasan et al., 2008).

We show that FLP-34 is required in serotonergic neurons for diacetyl learning, and food deprivation perceived by these neurons may trigger release of FLP-34. This role of FLP-34 neuropeptides would be consistent with the evolutionarily conserved role of NPY/NPF in hunger signaling and feeding (Sohn et al., 2013; Fadda et al., 2019). In mammals, modulation of feeding behavior by NPY is mainly restricted to hypothalamic nuclei, where Y1 and Y5 receptors stimulate orexigenic signaling under deprived conditions (Kohno and Yada, 2012). In *Drosophila* larvae, NPF signaling increases food-seeking behavior and inhibits locomotion to promote feeding under food-deprived circumstances (Wu et al., 2005). In *C. elegans*, NPR-11 in AIA has been shown to modulate local search behavior when worms are removed from food (Chalasani et al., 2010), which is reminiscent of NPFF-regulated food-seeking behavior in starved *Drosophila*. Diacetyl and food deprivation cues thus seem to converge on AIA interneurons expressing the NPYR/NPFR ortholog NPR-11.

In addition to FLP-34, our results suggest that serotonin modulates diacetyl learning, which is in agreement with its role as a reinforcement signal in aversive associative learning both in *Drosophila* and *C. elegans* (Giurfa, 2006). Based on the reported role of serotonin in signaling feeding state (Srinivasan et al., 2008; Donovan and Tecott, 2013; Voigt and Fink, 2015) and its action as a reinforcement signal in negative associations (Giurfa, 2006; Sitaraman et al., 2017), serotonin signaling may also reinforce the food deprivation cue in diacetyl learning. Although its target in this pathway remains unknown, the serotonin-gated chloride channel MOD-1 (Ranganathan et al., 2000), expressed in AIA as well as in most of the first layer interneurons of *C. elegans*, may be a promising candidate (Fig. 8B) (Harris et al., 2009). In another aversive learning paradigm where pathogenic bacteria serve as the aversive cue, MOD-1 activation on serotonergic neurons mediates olfactory learning in *C. elegans*. Cell 74:515–527.


